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BOTULINUM NEUROTOXIN AND TETANUS TOXIN

EDITED BY

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CHAPTER 1

Bacterial Sources of Clostridial Neurotoxins

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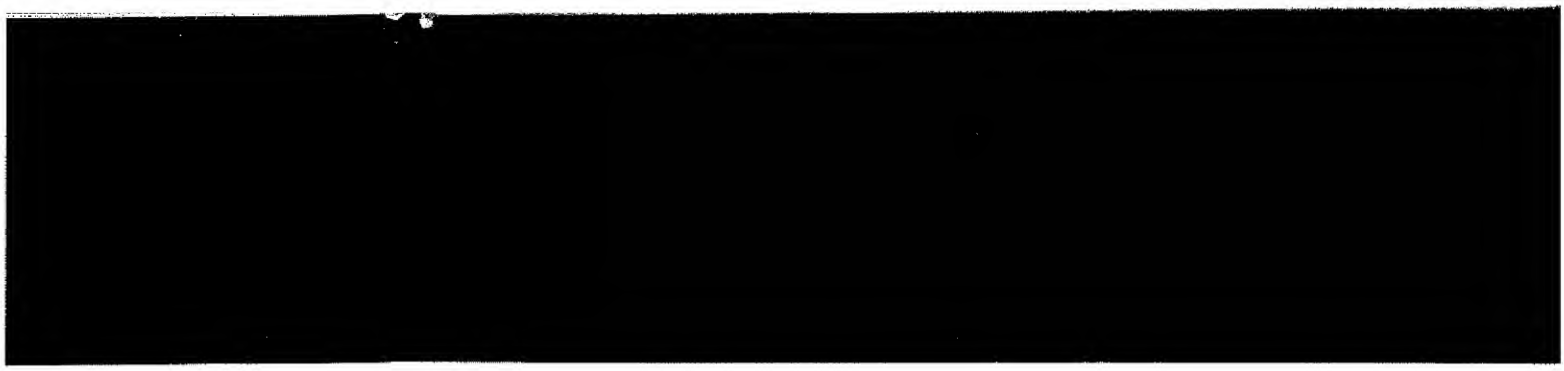

I. Introduction

The organisms capable of producing botulinum and tetanus neurotoxins belong to the genus *Clostridium*. Clostridia are anaerobic, spore-forming rods that are considered gram-positive, although some species do not actually show the positive Gram stain reaction. Some are gram-positive in young cultures but gram-negative in older cultures, while others show a mixture of positively and negatively stained cells. The most striking morphological feature is the spore, which since it is usually wider in diameter than the cell itself, causes the cell to swell and gives rise to the spindle shape to which the name *Clostridium* refers (*closter*, a Greek word meaning *spindle*).

Anaerobic organisms, according to one definition, "(i) generate energy and synthesize their substance without recourse to molecular oxygen; and (ii) demonstrate a singular degree of adverse oxygen-sensitivity which renders them unable to grow under an atmosphere of air" (Morris, 1975). Some species of clostridia are aerotolerant; they will grow on agar surfaces in the presence of air (Smith and Williams, 1984). They can be distinguished from members of the genus *Bacillus* by their lack of catalase. Clostridia lack cytochrome oxidase, which is involved in aerobic metabolism, and also lack the enzymes catalase and peroxidase, which protect biological systems against the deleterious effects of oxygen and oxygen derivatives (Decker *et al.*, 1970; Morris, 1975). Superoxide dismutase may be present in some obligate anaerobes, including clostridia (Hewitt and Morris, 1975). The most important nutrients used by anaerobes are monosaccharides, amino acids, unsaturated acids, keto acids, and alcohols, which are used in hydrogenation and dehydrogenation reactions yielding energy conserved by substrate level phosphorylations. Saturated fatty acids cannot be used to produce energy in the absence of oxygen for thermodynamic and mechanistic reasons (Decker *et al.*, 1970). These compounds are generally found as metabolic end products.

Clostridia are found in soils; sediments in lakes, rivers, and marine waters; intestinal tracts of humans and animals; decaying animal carcasses and other organic matter; and in sewage, where there is an abundance of energy sources and an anaerobic environment. They are thus widely distributed in nature and can readily find their way into wounds. If a low oxidation–reduction potential in the tissue results from disruption of the circulation and the activity of facultative organisms, the clostridia can grow in the wound.

Currently, 83 species of *Clostridium* are listed in Bergey's Manual (Cato *et al.*, 1986). Fourteen species are considered pathogenic (Smith and Williams, 1984). Most of the pathogenic species are well noted for their ability to produce toxins that play a role in the pathology of various infections involving clostridia. Examples are local tissue destruction in gas gangrene, necrotic enteritis caused by *Clostridium perfringens* type C, and paralytic effects in tetanus and wound botulism.

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II. Discovery of Neurotoxigenic Clostridia

The recognition of neurotoxigenic bacteria occurred during investigation of the diseases tetanus and botulism. In 1867, Pirogov hypothesized that tetanus was caused by an infectious agent (Bytchenko, 1981). Seventeen years later, Rattone showed that tetanus could be transmitted from a human suffering from the disease to a rabbit by inoculating the rabbit with material from the patient's infected wound, while at the same time Nicolaier discovered that implantation of soil samples into mice, rabbits, and guinea pigs could cause symptoms of tetanus in these animals (Kobel and Marti, 1985). Nicolaier postulated that the clinical manifestations of tetanus were due to a poison produced in patients by organisms in soil that contaminated their wounds. The "drumstick" form of the organism and its spores was described by Rosenbach and Flugge, and the organism was first isolated in pure culture by Kitasato from a patient in 1889 (Bytchenko, 1981). Pure cultures and cell-free filtrates were shown to cause tetanus when injected into animals. Behring and Kitasato demonstrated that animals could be immunized with tetanus toxin modified by iodine trichloride and that the animals' serum contained neutralizing antibodies (Wilson and Miles, 1975).

Botulism, or sausage poisoning, had been documented since the end of the eighteenth century, but it was not until 100 years later that its bacterial etiology and its toxicopathic nature came to light. In 1895, an outbreak of botulism involving three fatal, ten very serious, and several milder cases occurred in Ellezelles, Belgium. The incident was investigated by Emile Pierre Marie van Ermengem, and a thorough report of his extensive study was published about 3 years later (van Ermengem, 1897). The outbreak was caused by a salt-cured, uncooked ham. Portions of macerated ham fed to mice, guinea pigs, and monkeys caused signs of illness indicative of botulism and subsequently resulted in death; feedings to rats and cats had little effect, but subcutaneous injection caused symptoms and death. Dogs and chickens were unaffected by feeding or injections. Filtrates of macerated ham had the same effect as the macerate itself. van Ermengem suspected that a bacterial agent was responsible for the toxin, as in tetanus and diphtheria. He consistently found an anaerobic sporulating bacillus in cultures of the ham as well as in a culture of spleen from one of the deceased victims of the outbreak. Cultures and culture filtrates of those organisms had the same effect on susceptible animals as did the ham macerates. The organism was named *Bacillus botulinus*.

While the bacteriology of tetanus has remained relatively simple, the experience with botulism has revealed a rather complicated picture, for a multiplicity of toxin types and a diversity of organisms capable of producing the botulinum toxins were discovered during the 90 years following van Ermengem's studies. Furthermore, it was eventually established that botulism could occur as a result of growth of the organism in wounds (Merson and Dowell, 1973) and because of

colonization of the intestine in infants (Midura and Arnon, 1976, Pickett *et al.*, 1976) and others (McCroskey and Hatheway, 1988).

Although the etiologic agent of tetanus was discovered first, it will be easier to make the presentation of its biologic features after first presenting the complexities of the group of organisms capable of producing botulinum toxins. This order of presentation will also be more consistent with the title of this book.

III. Organisms That Produce Botulinum Neurotoxin

A. Toxin Types

Thirteen years after the report of van Ermengem (1897), Leuchs (1910) reported that the toxin from an organism responsible for a subsequent outbreak of botulism (Landman, 1904) could be distinguished from that of van Ermengem's bacillus on the basis of differential neutralization with specific antitoxins. In 1919, type A and B toxin designations were established using strains isolated from outbreaks of human botulism in the United States (Burke, 1919). The van Ermengem and Landman strains were no longer available by that time, but it is probable that the former would correspond to type B and the latter to type A. The generic name *Clostridium* was adopted in place of *Bacillus* as recommended by the Committee on Classification of the Society of American Bacteriologists; *Clostridium* was chosen to include anaerobic spore-forming organisms, while *Bacillus* included only aerobic organisms (Bengtson, 1924). The species *Clostridium botulinum* was expanded to include a diversity of culturally distinct organisms that produce botulinum neurotoxin discovered over the next several decades (Smith and Williams, 1984).

Organisms that caused botulism in chickens in the United States (Bengtson, 1922) and in cattle in Australia (Seddon, 1922) were of a new type, designated type C; toxin from both strains was neutralized with the Bengtson strain antitoxin, while antitoxin to the Seddon strain neutralized only the homologous toxin. The two subtypes of type C toxin were designated C_α and C_β respectively (Gunnison and Meyer, 1929). An organism similar to the type C strains but producing yet another type of toxin caused an outbreak of botulism in cattle in South Africa; this organism was designated toxin type D (Meyer and Gunnison, 1928). Two human botulism outbreaks in 1934 resulting from contaminated fish in New York State (Hazen, 1937) and in the Ukraine (Kushnir *et al.*, 1937) were due to type E botulinum toxin (Gunnison *et al.*, 1936–1937). Type F botulinum toxin was recognized in 1958 in the investigation of a botulism outbreak in Denmark due to liver paste (Moller and Scheibel, 1960). Thus, botulinum toxins of type A through F and the organisms that produce them were discovered through the investigation of botulism outbreaks in humans (types A, B, E, and F) and animals (types C and D).

Type G botulinum toxin was encountered in low concentration in a soil survey culture by Gimenez and Ciccarelli (1970b). The toxigenic organism was

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recovered from the culture in spite of its low toxigenicity and lack of lipase activity, which ordinarily provides a recognizable marker for *C. botulinum*. Sonnabend *et al.* (1981) reported isolating *C. botulinum* type G from autopsy specimens, but thus far there has not been adequate evidence that type G botulism has occurred in humans, nor in animals except under experimental conditions.

Serologic analysis of the toxins of type C and D organisms revealed that multiple components are produced by these strains. Type C_α produces C_1, C_2, and a small amount of D toxin, while type D produces a major amount of D and a minor amount of C_1. Type C_β produces only C_2 toxin (Jansen, 1987). C_2 toxin is a binary toxin consisting of two separate molecules (55,000 and 105,000 Da, respectively) that are not covalently linked. It is not a neurotoxin but causes hypotension, hemorrhaging in the lungs, and a collection of fluid around the lungs and in the trachea (Simpson, 1982). The *in vivo* effects of C_2 toxin appear to be due to changes in membrane permeability or cellular secretion.

Variation in serologic characteristics of toxins from different strains of the same toxin type has been noted. Ciccarelli and Gimenez (1971) differentiated two groups of strains of type A toxin producers, one with toxin consuming a low level of antitoxin in the neutralization test and a second with a high antitoxin requirement for neutralization. A similar difference in toxins from type B organisms was noted in the study of an isolate from an infant with botulism (Hatheway *et al.*, 1981). A strain isolated from soil produced two identifiable types of toxin, about 93% type A and 7% type F (Gimenez and Ciccarelli, 1970a). Two strains that produce about 90% type B and 10% type F toxin have been isolated from infants with botulism (Hatheway and McCroskey, 1987).

Thus, seven serologic types of botulinum neurotoxin have been identified, and minor variations and combinations have been noted. The serologic spectrum of botulinum toxins is rather complex compared with tetanus toxin, which has a single serologic type.

B. Physiologic Differences among Organisms Producing Botulinum Neurotoxin

It was noted early that strains of organisms that produce botulinum toxin have variable cultural characteristics. Leuchs (1910) noted that the van Ermengem strain grew well at 18–25°C but poorly at 35–37°C, while the Landman bacillus grew better at the higher temperatures. Differences were noted in sugar fermentation between the two strains. Information on milk protein digestion for these two strains was confusing, and the strains were no longer available for study when systematic comparisons were being made. Bengtson (1924) compared proteolytic activities of American type A and type B strains with those of a European type B strain, and found the American strains of both types were proteolytic for meat, egg, and serum proteins while the European strain was negative for all three types of protein. The latter strain was judged to conform most closely to

van Ermengem's description of his original isolate. In Bengtson's study (1924), all seven type C strains were nonproteolytic. Characteristics of organisms that produce type D toxin coincide with those of type C organisms.

Organisms that produce type E toxin resemble the nonproteolytic European type B organisms in their lower temperature optimum, in the lower heat resistance of the spores, and in their lack of proteolytic activity. Although very few type F botulism cases have been recorded, environmental studies have yielded strains approximately equally divided between proteolytic and nonproteolytic groups. The discovery of the organism that produces type G toxin expanded the nomenclature dilemma by adding another organism with even more disparate characteristics than encountered with the previous types and groups: the type G organism is asaccharolytic, and negative for lipase which generally serves as a key characteristic for isolating botulinum toxin-producing organisms (Gimenez and Ciccarelli, 1970a). However, the name *Clostridium botulinum* was applied to this as well as to all other organisms capable of producing botulinum neurotoxin.

At this point, four physiologic groups of *C. botulinum* are recognized (Smith, 1977). Each is designated with a Roman numeral (I, II, III, IV; Table I). Group I organisms are usually referred to as "proteolytic," while Group II organisms are "saccharolytic" (Cato *et al.*, 1986). Type B and type F toxins can be produced by strains belonging to either Group I or Group II. Group III contains only organisms that produce type C or type D toxin. They may or may not digest complex proteins, but they are distinguished from both Groups I and II by production of significant amounts of propionic acid. Group IV consists of organisms that produce type G neurotoxin. Recently, classification of neurotoxigenic organisms has become even more complicated since strains of clostridial species, *Clostridium baratii* and *Clostridium butyricum*, that also produce botulinum neurotoxin have been isolated (Hall *et al.*, 1985; McCroskey *et al.*, 1986).

C. Related Nontoxigenic Organisms

Nontoxigenic clostridial species phenotypically related to Groups I, III, and IV are *Clostridium sporogenes*, *Clostridium novyi*, and *Clostridium subterminale*, respectively (Table I). Nontoxigenic organisms similar to Group II are encountered but have not been given a species name. The existence of these nontoxigenic organisms has engendered speculation that each group could be considered a separate species consisting of toxigenic and nontoxigenic strains.

D. Genetic Studies

The genetic relatedness of toxigenic strains of *C. botulinum* Groups I, II, and III and their nontoxigenic counterparts has been studied (Lee and Riemann, 1970a, 1970b; Nakamura *et al.*, 1977, 1983; Wu *et al.*, 1972). Suen (1986) recently completed a DNA hybridization study on *C. botulinum* type G (Group IV) and strains identified as *Clostridium subterminale* and *Clostridium hastiforme*. In

Table I Differential Characteristics for the Four Physiologic Groups of *Clostridium botulinum*

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Table I Differential Characteristics for the Four Physiologic Groups of *Clostridium botulinum*

Group	Type of Toxin	Milk Digestion	Glucose Fermentation	Lipase	Metabolic Products[a]		Phenotypically Related Clostridium
					Volatile	Nonvolatile	
I	A,B,F	+	+	+	A,iB,B,iV	PP	*C. sporogenes*
II	B,E,F	−	+	+	A,B		
III	C,D	±	+	+	A,P,B		*C. novyi*
IV	G	+	−	−	A,iB,B,iV	PA	*C. subterminale*

[a]Metabolic acid end products in peptone–yeast extract–glucose medium. A, acetic; P, propionic; iB, isobutyric; B, butyric; iV, isovaleric; PP, phenylpropionic; PA, phenylacetic.

general, all strains within Group I and Group II, regardless of toxin type, are closely related, while strains of the same or different toxin types belonging to different physiologic groups have low relatedness. Phenotypically similar nontoxigenic organisms may show high, intermediate, or low genetic relatedness to their toxigenic counterparts. It appears there is heterogeneity among strains identifiable as *C. sporogenes*. Some are closely related to Group I *C. botulinum* while others are not (Lee and Riemann, 1970a; Nakamura *et al.*, 1977; Wu *et al.*, 1972). The type strain of *C. sporogenes* is apparently among the latter (Suen *et al.*, 1986).

There appears to be some genetic diversity among strains in Group III (Lee and Riemann, 1970a; Nakamura *et al.*,1983). At least two genetic groups of toxigenic organisms are intermediately related to each other as well as to *C. novyi* types A and B, and *C. haemolyticum*. Suen (1986) found that nine strains of *C. botulinum* type G were all closely related to each other as well as to two strains identified as *C. subterminale* and one of *C. hastiforme;* they were unrelated to the type strains of those two species, however.

In summary, the four groups of *C. botulinum* are clearly distinguishable from each other genetically; within each physiologic group there may be different degrees of relatedness. In each group, phenotypically similar, nontoxigenic organisms exist, some of which may be closely related to some of the toxigenic organisms in that group.

E. Bacteriophages

Bacteriophages associated with Groups I, II, and III *C. botulinum* have been documented (Inoue and Iida, 1968; Eklund *et al.*, 1969; Dolman and Chang, 1972). Only with Group III strains has there been an association between phages and toxigenicity of the bacteria (see Chapter 2). Type C and D strains cured of their phages no longer produce toxin. Such nontoxigenic organisms could be converted to toxigenic forms, producing either type C or type D toxin, by reinfecting the organism with the phage isolated from the corresponding toxigenic strain (Eklund and Poysky, 1974). Subsequently it was demonstrated that a type C strain cured of its phage could be converted to an organism that was indistinguishable from *C. novyi* type A by infecting it with a phage (NA1) derived from a strain of *C. novyi* (Eklund *et al.*, 1974). Phage studies suggest that *C. botulinum* type C_β is derived from C_α after C_α loses the phage responsible for producing type C_1 toxin (Jansen, 1987).

F. Plasmids

Plasmid studies of *C. botulinum* have been pursued because of the interest in the genetics of toxigenicity in these organisms. Strom *et al.* (1984) isolated plasmids from all four groups of *C. botulinum*, including strains of all toxin types. Multiple plasmids were found in strains of Group I, types A and B, and Group III,

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Fig. 1. Photomicrographs of *Clostridium botulinum* and *Cl* indicated by bar in lower left (B) *Clostridium botulinum* typ *botulinum* type B, Group II (n type E (CDC5545). (E) *Clostr tetani* (CDC6202). Photograph

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types C and D. Single plasmids that differed between strains were found with Group II strains of types B and E. A single, uniform plasmid was found for Group I type F (11.5 MDa, five strains), Group II type F (2.2 MDa, four strains), and type G (73 MDa, six strains). No phenotypic functions were determined for the plasmids in that study. Weickert *et al.* (1986) studied 12 strains of *C. botulinum* type A with differing plasmid profiles and observed toxigenicity in organisms that initially lacked plasmids as well as in organisms whose plasmids had been eliminated after growth in medium containing sodium deoxycholate. Toxigenicity was lost by some of the isolates obtained after treatment, but there was no correlation with plasmid loss.

G. Cellular Morphology and Spores

The organisms in all four groups are medium to large rods, measuring 0.5 to 2.0 μm wide and 1.6 to 22.0 μm long (Cato *et al.*, 1986). They appear singly or in

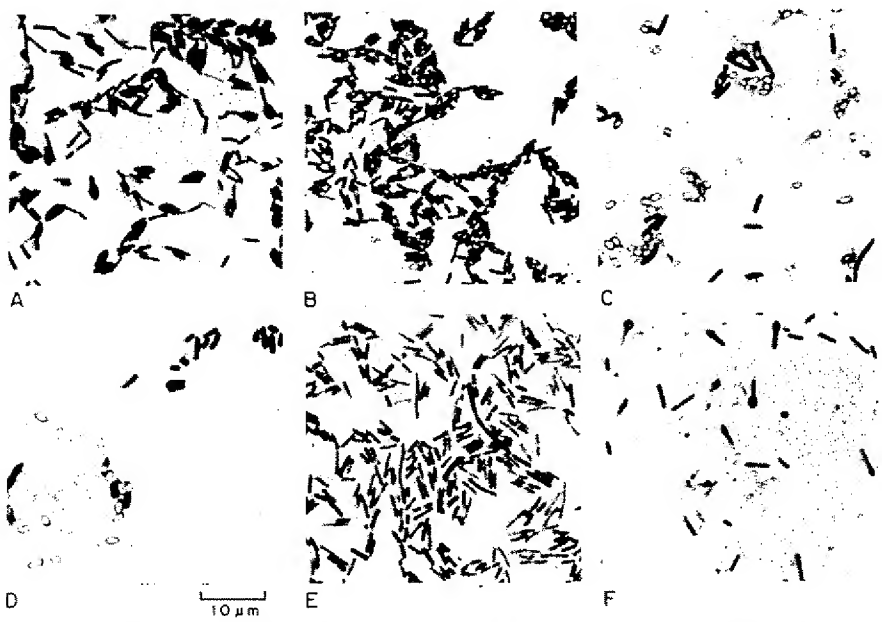

Fig. 1. Photomicrographs of gram-stained smears of egg yolk agar cultures of *Clostridium botulinum* and *Clostridium tetani*. Uniform magnification for all strains indicated by bar in lower left figure. (A) *Clostridium botulinum* type A (CDC5356). (B) *Clostridium botulinum* type B, Group I (proteolytic) (CDC5448). (C) *Clostridium botulinum* type B, Group II (nonproteolytic) (CDC5569). (D) *Clostridium botulinum* type E (CDC5545). (E) *Clostridium botulinum* type G (CDC1353). (F) *Clostridium tetani* (CDC6202). Photography by Don Howard.

pairs and are variable in their Gram-stain reactions. Spores are subterminal and are larger in diameter than the cell, thus causing swelling of the rod in the area of the spore. Photomicrographs of some representative strains are shown in Fig. 1 and 2. Available strains of *C. botulinum* type G do not sporulate readily, and usually no spores are seen on smears of those cultures. However, Lynt *et al.* (1984) succeeded in producing spore crops from two strains for heat resistance studies.

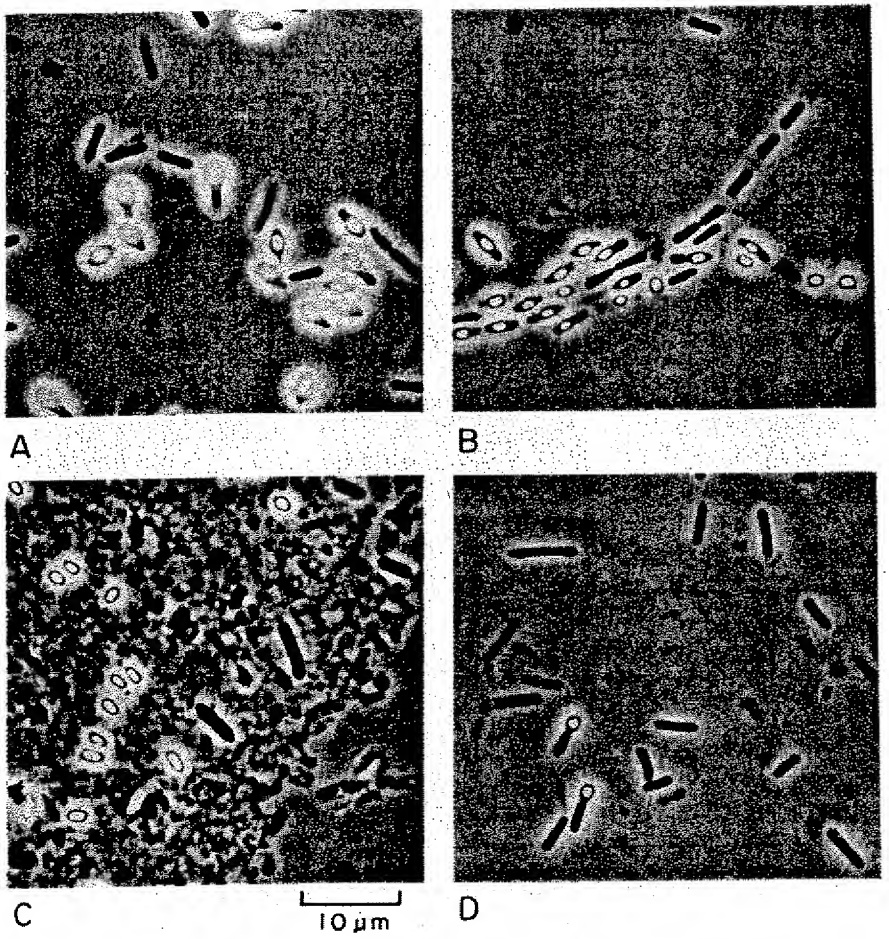

Fig. 2. Photomicrographs of phase-contrast views of wet-mount suspensions of egg yolk agar cultures of *Clostridium botulinum* and *Clostridium tetani*. Uniform magnifications for all strains indicated by bar in lower left picture. (A) *Clostridium botulinum* type A (CDC5356). (B) *Clostridium botulinum* type B, Group I (proteolytic) (CDC5448). (C) *Clostridium botulinum* type E (CDC5545). (D) *Clostridium tetani* (CDC6203). Photography by Don Howard.

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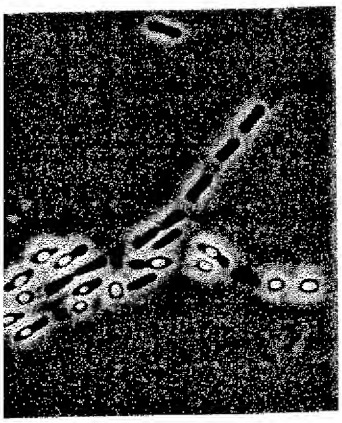

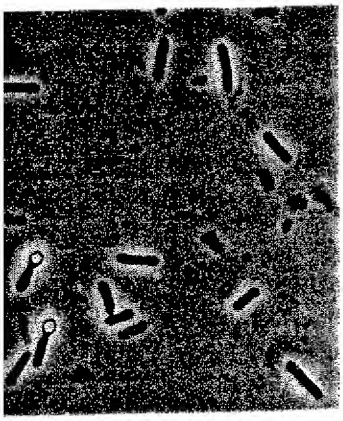

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When spores are not visible on stained smears, their presence may be shown by survival after heat or alcohol treatments designed to destroy vegetative organisms. Group I spores are heat resistant; many strains maintain viability after treatment in a boiling waterbath for 2 hr. Group II spores are not, often failing to survive 80°C for 10 min. Alcohol treatment is recommended for selecting spores of Group II organisms. Group III spores have a heat resistance intermediate between those of Groups I and II. *C. botulinum* type G appears to produce two populations of spores: 99% or more have a heat liability similar to spores of Group II; the remainder show heat resistance comparable to that of Group I spores (Lynt *et al.*, 1984).

H. Cultural and Physiologic Characteristics

All four groups of organisms are strict anaerobes; they will not grow on the surface of agar medium in ambient atmosphere or in a candle jar. They form discrete colonies on agar surfaces, and the lipase reaction, detectable on egg yolk medium, is the primary differential characteristic of all except type G. Key characteristics are shown in Table I. The optimum growth temperature range for Groups I, III, and IV is approximately 30° to 37°C, with good growth at 45°C. Group II organisms have a lower optimum temperature range, 25° to 30°C, with little or no growth at 45°C; a minimum growth temperature as low as 3.3°C has been reported (Eklund *et al.*, 1967).

I. Other Clostridia That Produce Botulinum Neurotoxin

Toxigenic organisms responsible for three cases of infant botulism resembled clostridial species unrelated to the four groups of *C. botulinum*. The organism isolated from an infant with type F botulism in New Mexico resembled *C. baratii* (Hall *et al.*, 1985). Organisms responsible for two cases of type E infant botulism in Italy were identical in all phenotypic characteristics except their toxigenicity to *C. butyricum* (Aureli *et al.*, 1986; McCroskey *et al.*, 1986). Subsequent DNA hybridization studies showed that the toxigenic isolates were closely related to the type strains of the species they resembled (Suen *et al.*, 1988). The New Mexico isolate was 86% related to *C. baratii* ATCC 27638, with no more than 3.5% divergence (unpaired nucleotides within related sequences). The isolate from the first Italian type E case was 78% related to *C. butyricum* ATCC 19398, with 1% divergence.

J. Habitat and Distribution

C. botulinum is found in various frequencies in soils as well as in sediments in lakes and seas in most parts of the world (Hauschild, 1989). Soil surveys in the United States have shown widespread distribution, with most isolates being either type A or B (most likely all Group I), with the former predominating in the

western half of the country and the latter in the east (Smith, 1978). The organisms are generally not found in normal human feces (Easton and Meyer, 1924; Dowell *et al.*, 1977b). They are occasionally found in feces of normal farm animals but do not appear to be a significant part of the normal flora (Easton and Meyer, 1924). Type C organisms are often present in carrion and thus are responsible for epizootics. Poultry by-products used as feed, bedding, and grassland fertilizer have been implicated as sources of outbreaks in cattle (Egyed, 1987; Smart *et al.*, 1987).

K. Toxin Detection and Identification

The most reliable means of detecting and identifying botulinum toxin in cultures and extracts from unknown specimens is by using mouse toxicity and neutralization tests (Hatheway, 1988). Samples (0.4 ml) of culture supernatants or filtrates, or extracts of specimens, are injected intraperitoneally into mice (approximately 20 g) with and without monospecific and polyvalent botulinum antitoxins. Mice injected with unneutralized neurotoxin die within 6–48 hr, depending on dose. Signs of botulism in the mice can be seen before death. The toxin type is established by observing which specific antitoxin neutralizes the toxicity. *In vitro* toxin assays may be more applicable when assaying numerous samples of toxin from a known source, for example, samples of fractions in a chromatographic purification process. The various procedures for assaying the toxin and merits of these various procedures are discussed more fully in Chapter 14.

L. Isolation of Organisms

Recovery of *C. botulinum* for clinical specimens and food and soil samples is relatively easy because of the possibility of eliminating much of the competing flora by spore selection and because of the differential characteristic lipase. Selective egg yolk agar media can also be helpful (Dezfulian *et al.*, 1981; Mills *et al.*, 1985). Heat treatment is effective for recovering heat-resistant spores. To recover spores of Group II organisms, either a lower temperature for heat treatment or alcohol treatment (Johnston *et al.*, 1962) is sometimes necessary. Occasionally it is difficult to isolate toxigenic organisms because nontoxigenic, lipase-positive organisms such as *C. sporogenes* are also present in high numbers. An antitoxin overlay immunodiffusion method has been proposed to distinguish between colonies of toxigenic and nontoxigenic organisms on agar plates (Ferreira *et al.*, 1983). Initial cultures are inoculated into chopped meat or chopped meat–glucose–starch medium (Dowell *et al.*, 1977a); organisms are isolated by streaking enrichment cultures or suspensions of the original specimen on selective or nonselective egg yolk agar (Hatheway, 1988). Single colonies are picked from the agar surface and inoculated into chopped meat–glucose–starch medium. Toxigenicity is established by mouse toxicity and neutralization tests on culture supernatants. Enrichment cultures should also be tested for toxin,

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especially since lipase-negative organisms such as *C. botulinum* type G, *C. baratii*, and *C. butyricum* can produce neurotoxin. Isolated organisms can be fully characterized using methods described by Dowell and Hawkins (1974), Holdeman *et al.* (1977), and Dezfulian and Dowell (1980).

IV. Organisms That Produce Tetanus Neurotoxin

A. Toxins Produced by *Clostridium tetani*

The neurotoxin produced by *C. tetani* is referred to as tetanospasmin. Only one serologic type of tetanospasmin is known, and the organisms that produce it are phenotypically similar. Based on cellular antigens, nine bacterial agglutination types have been recognized (Coleman and Gunnison, 1928; Smith and Williams, 1984).

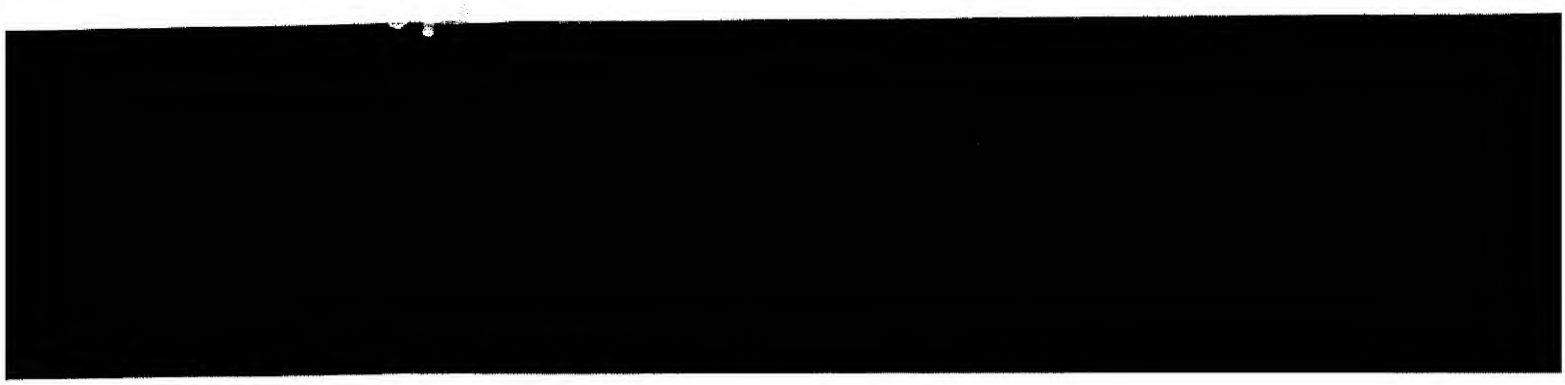
Physiologic characteristics of *C. tetani* strains are fairly uniform. Some strains of *C. tetani* that are toxigenic when initially isolated subsequently lose their toxigenicity (Fildes, 1925). A second toxin, tetanolysin, causes hemolysis on blood agar plates. It is doubtful that this toxin plays any role in the pathology of tetanus (Adams *et al.*, 1969).

B. Cellular Morphology and Spores

C. tetani is usually a gram-negative rod that may retain some gram-positive staining in portions near the spore. The entire cell may be gram-positive in young cultures (less than 18 hr old). Unsporulated cells in cultures older than 24 hr usually appear gram-negative. Cell dimensions are 0.5–1.7 × 2.1–18.1 μm. Most strains are motile by means of peritrichous flagella (Cato *et al.*, 1986). Spores are usually in a terminal position and are more common in older cultures. Photomicrographs of representative strains are shown in Fig. 1 and 2. Spores generally survive moderate heating (75–80°C for 10 min) but usually are destroyed after heating at 100°C for 1 hr. Spores of some strains are more resistant, so that heating at 100°C for 4 hr or autoclaving for at least 10 min is required to ensure sterility (Adams *et al.*, 1969). Spores can survive exposure to alcohol, phenol, and even formaldehyde solutions. Aqueous and alcoholic iodine, gluturaldehyde, and hydrogen peroxide are some of the more effective sporicidal agents.

C. Cultural and Physiologic Characteristics

C. tetani is a strict anaerobe. It will grow on the surface of solid media such as blood agar, peptone agar, and egg yolk agar only in an anaerobic environment



such as a Gas-Pak jar (BBL Laboratories, Cockeysville, Maryland)* or in an anaerobe chamber with an atmosphere of 10% H_2, 5% CO_2, and 85% N_2. Optimal growth occurs at 37°C, while little or no growth takes place at 25°C or 42°C. Growth may not be visible until after 48 hr of incubation. Growth on agar is often apparent as a film rather than as discrete colonies because of swarming due to vigorous motility. Media containing 3–4% agar are more conducive to formation of discrete colonies. Colonies on blood agar are 4–6 mm in diameter, flat, translucent, gray with a matte surface, and show a narrow zone of clear (beta-type) hemolysis. Colonies have irregular and rhizoid margins (Cato *et al.*, 1986).

Growth in liquid medium does not require incubation in an anaerobic atmosphere. Dissolved oxygen can be driven out by heating tubed media in a boiling waterbath for 10 min. The broth is cooled before being inoculated. Metallic iron (filings or strips) or sodium thioglycollate help produce reducing conditions. *C. tetani* spores can germinate in media with an oxidation–reduction potential E_h of +580 mv, although vegetative growth does not occur unless the E_h is diminished. It has been shown that the E_h of liver broth diminishes after inoculation of spores sufficient for vegetative growth if the initial E_h is +300 mv or less, or even at +500 mv if the medium has a cover of liquid paraffin (Hachisuka *et al.*, 1982).

Most common biochemical tests are negative. No sugars are fermented; milk and other complex proteins are not digested. Neither lecithinase nor lipase is produced, and nitrate is not reduced. Gelatin is liquefied slowly, requiring perhaps 7 days for complete liquefaction. Hydrogen sulfide, DNase, and indole are produced. Gas-liquid chromatography of cultures in peptone-yeast extract with or without glucose (Lombard and Dowell, 1982) show butanol and acetic, propionic, and butyric acids as metabolic end-products (Holdeman *et al.*, 1977).

D. Genetic Studies

Nakamura *et al.* (1979) compared 10 strains of *C. tetani* and found they were all related to each other by 89% DNA sequence similarity. Three nontoxigenic strains (*C. tetani*-like strains that resembled *C. tetani* in all respects except toxigenicity) were closely related (85–93% similarity). *C. tetani* could be clearly distinguished from *C. cochlearium* and *C. tetanomorphum*, which are nontoxigenic, by DNA sequence differences. Oddly, one *C. tetani*-like strain that was toxigenic when isolated but lost toxigenicity after treatment at 100°C for 30 min was more closely related to *C. cochlearium* than to *C. tetani*. Cato *et al.* (1986) distinguished between *C. cochlearium* and *C. tetani* on the basis that the former is nontoxigenic and is weaker or negative in the liquefaction of gelatin. Thus it

*Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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seems very difficult to distinguish between nontoxigenic *C. tetani* and *C. cochlearium* without DNA comparison. Soluble cellular proteins of the two species may be distinct (Cato *et al.*, 1982), but this has not been verified by comparing a sufficient number of strains identified by DNA studies. The species *C. tetanomorphum* has been omitted from Bergey's Manual because the available reference strains do not have the characteristics originally described for the species (Cato *et al.*, 1986). *C. tetani* has 25–26 mol% G+C content in its DNA, while *C. cochlearium* has 27–28% (Cato *et al.*, 1986).

E. Bacteriophages, Plasmids, and Toxigenicity

Based on the findings of the relation of a lysogenic phage to toxigenicity of *Corynebacterium diphtheriae*, researchers sought evidence for a phage role in toxin production by *C. tetani*. Prescott and Altenbern (1967a) found that mitomycin C induced lysis of all seven strains of *C. tetani* tested. This provided presumptive evidence of lysogenic phages, but there was no indication that any of the possibly induced phages were related to toxigenicity. The presence of phages in two of the strains was verified by electron microscopy (Prescott and Altenbern, 1967b). Hara *et al.* (1977) cured a strain of *C. tetani* of its toxigenicity by treating it with acridine orange, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, rifampicin, and ultraviolet light; they found that the nontoxigenic derivative strains appeared to possess the same bacteriophages as the toxigenic parent strain. Subsequently, studies on toxigenic strains of *C. tetani* and nontoxigenic derivatives of three toxigenic strains revealed that loss of toxigenicity was correlated with loss of a single large plasmid (Laird *et al.*, 1980). Further investigation using oligonucleotides coding for an amino-terminal amino acid sequence in hybridization reactions showed that the gene for the toxin is on the plasmid (Finn *et al.*, 1984). The entire nucleotide sequence of the plasmid toxin gene and the amino acid sequence and structure of the toxin molecule have since been reported (Eisel *et al.*, 1986).

F. Habitat and Distribution

C. tetani is commonly found in soil samples in all parts of the world where surveys have been made. Surveys in Japan, Canada, Brazil, and the United States have yielded 30–42% positive samples (Smith and Williams, 1984). *C. tetani* has also been commonly found in human and animal feces. Bauer and Meyer (1926), Ten Broeck and Bauer (1922), and Tulloch (1919–1920) found the organism in about 25–35% of human fecal samples in the United States, China, and England, respectively. Intestinal carriage may be responsible for naturally acquired immunity to tetanus toxin in some nonimmunized humans and animals (Veronesi *et al.*, 1983). Antitoxin, but no toxin, has been found in the serum of experimental animals that have been intestinally colonized with *C. tetani* (Wells and Balish, 1983).

G. Toxin Detection and Identification

Tetanus toxin can be demonstrated by intramuscular (im) injection of the test sample into a test animal. For example, 0.1 ml of a culture supernatant is injected into the rear leg muscle of a mouse; the mouse is observed for several days for the development of rigid paralysis in the injected muscle and the progression of the paralysis into the rest of the musculature of the lower body. A second mouse injected with a mixture of 0.1 ml of culture supernatant and 0.1 ml of tetanus antitoxin should show no paralytic signs. Intraperitoneal (ip) injection of moderate to large amounts of toxin may kill the mice before signs of tetanus can be recognized; smaller amounts cause a distended, rigid abdominal area with gradual development of a generalized tetanus. Injection of samples mixed with antitoxin should have no effect, unless the amount of toxin overwhelms the neutralizing capacity of the antitoxin. Beland and Rossier (1973) recommend testing cultures for toxigenicity by mixing equal volumes of broth culture and 2% calcium chloride and injecting 0.5 ml of this mixture at the base of the tail of each mouse. Mice pretreated by ip injection of 1500 IU of tetanus antitoxin 1 hr before challenge serve as controls. Test animals should be properly disposed of as soon as a conclusive judgment can be made on the basis of paralytic signs.

H. Isolation of Organisms

Tetanus is not usually confirmed bacteriologically because the signs of this condition provide unmistakable evidence for the diagnosis. Often the infected wound is not obvious, and when cultured, the causative organism is not recovered. Most of the strains of *C. tetani* have been isolated from soil. *C. tetani* can often be isolated by culture from material that does not show typical "drumstick" organisms upon microscopic examination (Adams *et al.*, 1969). Pieces of dead tissue taken from deep in wounds are ideal specimens for culture. Swabs of the wound offer less chance for success. Soil samples can be cultured using the same methods used for wound samples.

The medium of choice for culturing *C. tetani* is chopped meat medium (Dowell *et al.*, 1977a). Selective advantage is taken of the spores' resistance to heat, although heat treatment may result in decreased toxicity of the subsequent cultures and increased numbers of nontoxigenic isolates. Adams *et al.* (1969) recommend inoculation of three broth cultures, heating one at 80°C for 15 min, heating the second for 5 min, and not heating the third. Beland and Rossier (1973) recommend inoculating soil samples after suspending them in sterile saline: one portion of the suspended sample is inoculated after a 10 min treatment at 60°C; another portion is inoculated without treatment. Both cultures after 48 hr incubation at 37°C are streaked onto a half (or less) of a blood agar plate and incubated anaerobically at 37°C and observed at 24 and 48 hr for swarming growth. The bacterial growth at the leading edge of the swarming growth is picked and inoculated into chopped meat medium. Distinct colonies of the

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V. Final Considerations

In view of the unusual features of some of the toxigenic organisms that have caused botulism, organisms responsible for tetanus cases should be isolated when possible. Reports of tetanus in patients with demonstrable circulating tetanus antitoxin (Berger *et al.*, 1978; Passen and Andersen, 1986) suggest the possible existence of strains that produce a type of tetanospasmin that is not neutralized by the antibodies engendered by the immunizing toxoid.

The observation of production of botulinum neurotoxin in strains of *C. baratii* and *C. butyricum* raises the question of whether it is practical to call all organisms that produce the toxin *C. botulinum*. Additionally, nontoxigenic organisms, which may have been derived from toxigenic strains, cannot be properly called *C. botulinum*. Thus, strict classification of *Clostridim* species on the basis of toxigenicity seems to deserve reconsideration.

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